Synergistic interactions of polyphenols and their effect on antiradical potential

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Abstract: The aim of the current study was to evaluate interactions among polyphenols from different plants and their effect on antioxidant potential. Different mixtures of plant extracts of *Crataegus oxyacantha* (C), *Elettaria cardamomum* (Cr), *Terminalia arjuna* (T) and *Rauvolfia serpentina* (R) were prepared and evaluated for total phenolics, flavonoid contents, and antioxidant activity. A correlation was also established between total phenolics, flavonoids and antioxidant activity. Comparative evaluation revealed that phenolics, flavonoids and antioxidant activity were found high in plant extracts mixtures than individual plants. Highest phenolics (580±1.12mg GAE/g), flavonoids (67.10±0.11mg CE/g) and antioxidant activity (IC_{50} 0.109mg/ml) was observed with ratio 1:1:1:2 of plant mixture C, Cr, T, R. A weak linear positive correlation was found between antioxidant activity, total phenolic and flavonoid contents. A negative correlation was observed among IC_{50} value, total phenolics and flavonoid contents. Investigation through RP-HPLC revealed the presence of different potent phenolics in plants understudy. More antioxidant potential of extracts in combinations as compared to that of individual plants was clear corroboration of synergism. The ratio (1:1:1:2) of the studied plants in combination, that showed the highest free radical potential, was another expected better pharmacological prospect. This formulation can bring maximum relief against free radical-associated diseases.

Keywords: Plant combination, synergism, correlation, HPLC, DPPH assay.

INTRODUCTION

Bioactive components of plants have gained attention in recent years due to their significant role in the retardation of many chronic degenerative diseases. Phenolic and flavonoids are documented as the major active components responsible for the antiradical potency of many herbs. Interactions among these bioactive constituents from different plants may enhance their therapeutic potential and minimize their side effect to a great extent (Jankobek *et al*., 2011). Many conventionally used herbs show appreciably better pharmacological properties when used in combination than used alone (Altunkaya *et al*., 2009). Single plant due to limited bioactive compound may not combat against complex diseases (Zimmermann *et al*., 2007). Botanical therapeutics with multi-component have numerous benefits over single plant extract/isolated compound that may give them a more prominent place in the field of herbal medicines (Nedamani *et al*., 2014).

Medicinal plants may serve as a source of valuable lead compounds. For pharmaceutical exploitation, it is required to isolate these lead compounds and to determine their bioactivity. Analytical techniques play significant roles in the invention, advancement and production of pharmaceuticals (Ramya *et al*., 2010; Mariswamy *et al*., 2011). Traditional remedial system recognized several plants that have free radical quenching properties owing to the presence of phytochemicals. *T. arjuna* is a tree belonging to family *Combretaceae*. Its different parts (stem, leaves, and bark) are rich source of flavonoids, triterpenoids, glycosides, tannins, saponins and minerals (Nema *et al*., 2012). *R. serpentina* (family *Apocynaceae*) root contains almost 50 indole alkaloids. Its reserpine phytochemical is a potent sedative (Deshmukh *et al*., 2012). *E. cardamomum* has well-known culinary values. Its seeds are used in traditional system of medicines for the treatment of cardiac disorders, gastrointestinal disorders, nausea, influenza, diarrhea, asthma, bronchitis and cataracts (Verma *et al*., 2009). *C. oxyacantha* flowers, leaves and berries possess a diversity of flavonoid compounds that are accountable for the health benefits of this plant (Tadic *et al*., 2008).

Toxicity, resistance and financial load of current medication except those of herbal origin demand the invention of new remedies that will be helpful for the civilization (Dubey *et al*., 2004). To explore the new sources of antioxidants in recent years, medicinal plants have been widely investigated and found to be enriched with antioxidants. Combination of different plants is often more useful than individual plant in medication. In accordance with conventional medicinal system, a mixture of substances is used to boost the required action and to get rid of unnecessary side effects (Prince *et al*., 2008). The innate occurrence of antioxidants in plants and a mixture with other antioxidants possibly may have a

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synergistic effect. No significant data on interactions of antioxidants in various plant extracts to study synergism is presently available. To get insights about interactions and antiradical potential, a comparative study of four medicinal plants and their sixteen combinations was made.

MATERIALS AND METHODS

Collection of plants

Four medicinally important plants were selected for this study as follows: *T. arjuna* (bark), *R. serpentina* (roots), *E. cardamomum* (fruit) and *C. oxyacantha* (fruit). *T. arjuna* was collected from University of Agriculture, Faisalabad and identified by a plant taxonomist from Department of Botany, University of Agriculture, Faisalabad. Remaining plants were collected from local market of Faisalabad.

Preparation of different mixtures of plants

Sixteen mixtures of these selected plants were made by varying the ratio. These mixtures are shown in table 1.

Reflux extraction

Extracts of selected plants part were prepared to elicit phyto-constituents of plants. *C. oxyacantha* methanolic extract was purchased from local market. Other plants parts were air dried and grinded to powder form. Powdered plant material was elicited with methanol in a reflux apparatus for 1 hour (plant versus solvent 1:10 ratio). After extraction, they were filtered, concentrated in a rotary evaporator (Rotavapor R-II Buchi, Switzerland) and stored in a refrigerator for further analysis (Aslam *et al*., 2012; Anwar *et al*., 2013).

Determination of total phenolic contents

Total phenolic contents of plants and their mixture were ascertained. One ml of plant extract (0.0001g/ml) was mixed with Folin-Ciocalteu reagent (5ml, twenty times diluted) and sodium carbonate (4ml, 20%). After incubation for an hour, the absorbance of resulting blue color complex was noted at 765nm with UV-vis spectrophotometer (DB-20, Australia). Gallic acid standard curve (0.01-0.1 mg/ml) was also plotted in same manner and concentration of phenols in plant extract was estimated from standard curve (Pourmorad *et al*., 2006).

Phenolics fingerprinting by RP-HPLC

HPLC system for phenolic acid analysis was consisted of Shim-pack CLC-ODS (C-18) column (25cm × 4.6mm, 5µm) and gradient mobile phase. Solvent A in mobile phase was consisted of H_2O: Acetic acid (94:6, pH=2.27) and solvent B acetonitrile (100%). Flow rate was set 1ml/min and 0-15min=15% B, 15-30min = 45%B, 30-45min= 100% B. Detection was carried out with UV-visible detector (λ max 280 nm). The hydrolysis of the test samples was carried out to get free phenolics. In brief, extract (50mg) was dissolved in methanol (24mL) and homogenized. Distilled water (16ml) was then added followed by HCl (10ml, 6M). The mixture was then incubated for 2 hr at 95ºC. Final solution was filtered using a 0.45µm nylon membrane filter prior to high performance liquid chromatography (HPLC) analysis (Hertog *et al*., 1992).

Estimation of total flavonoid contents

Aluminum chloride colorimetric method was used for the estimation of flavonoid contents in plant extracts. Various concentrations of standard compound Catechin (0.02-0.1 mg/ml) were prepared. Aliquot (1ml) of these concentrations was taken and mixed with distilled water (3ml) and 0.3ml of $NaNO_2$ (5%). After 5 min at 25°C, 3 ml of $AlCl_3$ (10%) was added. After further 5 min, the reaction mixture was treated with 2 ml of NaOH (1mM). Finally, the reaction mixture was diluted up to 10ml with water. Absorbance of resulting solutions was measured at 510 nm with UV-vis spectrophotometer (DB-20, Australia (Eghdami and Sadeghi, 2010).

Determination of antioxidant activity (AA)

The antioxidant activity of all plant extracts were evaluated individually and in mixture by DPPH free radical scavenging assay. Different concentrations of plant extracts (0.1-1mg/ml) were prepared and mixed with 1ml DPPH (0.1mM). Reaction mixture was incubated at 35ºC for 30 min. A blank solution was also run similarly with distilled water (3ml) as control. After incubation, the purple color of DPPH developed which was read at 517nm using a UV-vis spectrophotometer (DB-20, Australia) (Proestos *et al*., 2013).

STATISTICAL ANALYSIS

All assessments were performed in triplicate and data were presented with mean ± standard error of mean. Data were analyzed by one way ANOVA followed by Tukey's multiple comparison tests. $P \leq 0.05$ was considered as significant. The data was correlated using Pearson correlation coefficient (r). The IC_{50} values were calculated using linear regression analysis.

RESULTS

Determination of total phenolic contents (TPC)

Comparative study of total phenolic contents (TPC) phenolic contents revealed the presence of greater phenolic contents in plant mixtures than single plant extract. Among individual plant extracts, *T. arjuna* exhibited the presence of highest phenolic contents followed by *R. serpentina*, *C. oxyacantha* and *E. cardamomum*. Among plant extract mixtures, the highest TPC were observed in 8^{th} mixture (T: R: C: Cr, 1:2:1:1) followed by 6^{th} mixture (T: R: C: Cr, 4:4:2:1). The lowest amount of phenolics was observed in 14^{th} mixture (fig. 1).

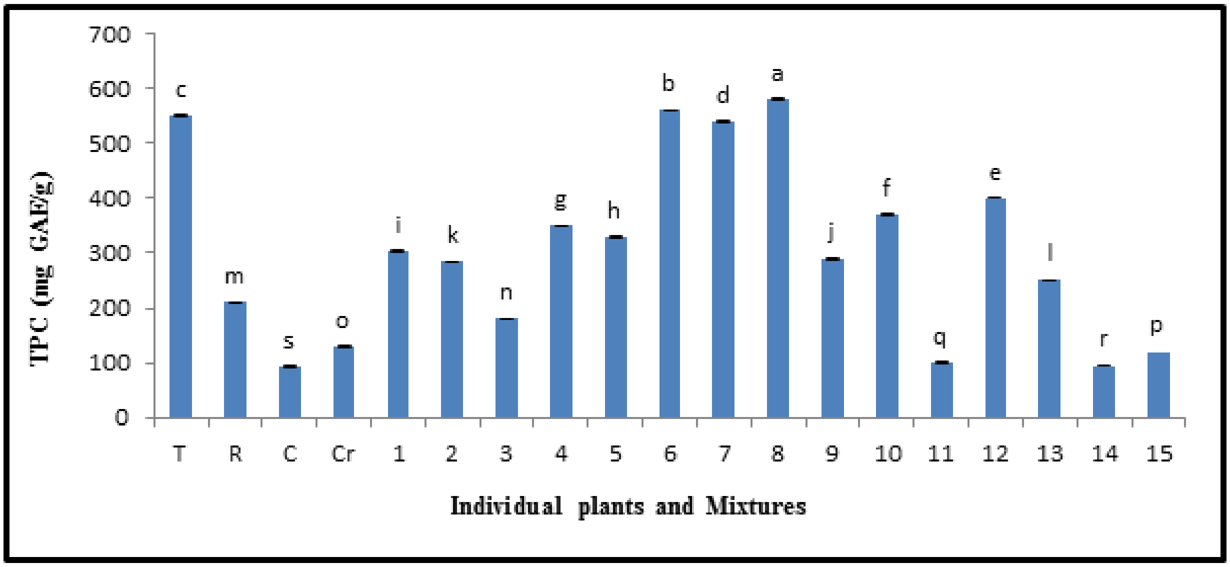

Fig. 1: Total phenolic contents of individual plants and their mixtures. Error bars indicate mean ± SD (n=3). Means that do not share common letter are significantly different from each other (P≤0.05).

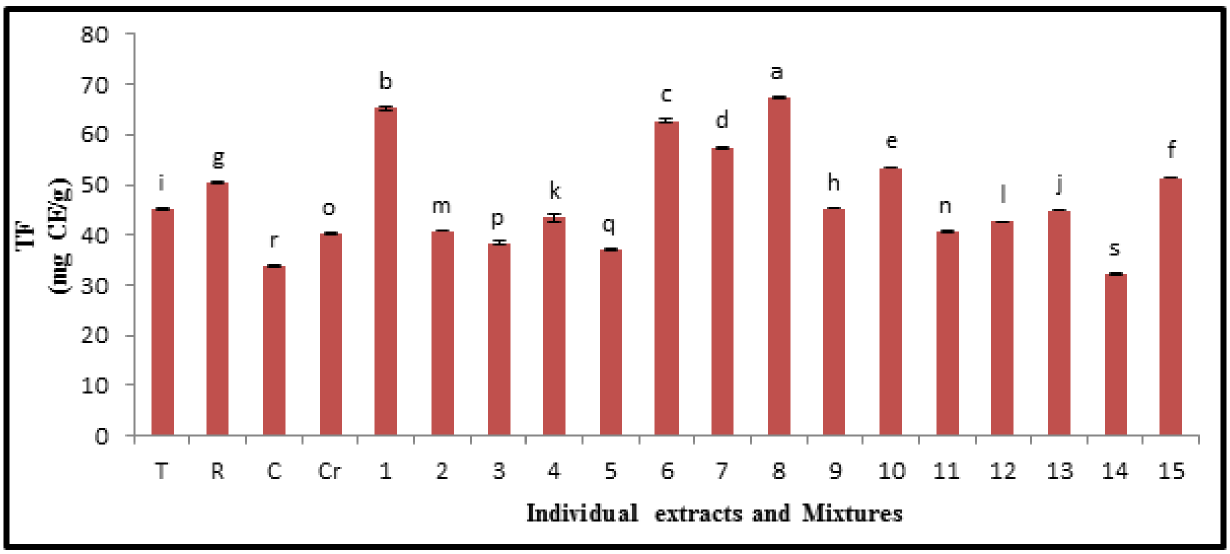

Fig. 2: Total flavonoid contents of individual plant extracts and their mixture. Error bars indicate mean ± SD (n=3). Means that do not share common letter are significantly different from each other (P ≤ 0.05).

Phenolics fingerprinting by RP-HPLC

Five phenolic acids identified in medicinal plants by HPLC analysis were p-coumaric acid, caffeic acid, vanillic acid, chlorogenic acid and ferulic acid. Their respective concentrations in each plant have been shown in table 2. Highest number of phenolic acids were identified in *C. oxyacantha* (four) followed by *E. cardamomum* (three) and *R. serpentina, T. arjuna* (two). *R. serpentina* showed the presence of caffeic acid and p-coumaric acid while vanillic acid, chlorogenic acid and ferulic acid were not identified in this plant. *C. oxyacantha* showed the presence caffeic acid, chlorogenic acid, p-coumaric acid and ferulic acid. Vanillic acid was not identified in any plant except *E. cardamomum, T. arjuna* revealed the occurrence of chlorogenic acid in highest concentration. Beside this other phenolics determined was ferulic acid. Caffeic Acid, vanillic acid and p- coumaric acid were not identified contrasted to other plants. *E. cardamomum* showed the presence of ferulic acid in highest concentration followed by vanillic acid and caffeic acid. Chlorogenic acid and p- coumaric acid were not identified in this plant compared to other plants.

Estimation of total flavonoid contents (TF)

Significant differences were observed in total flavonoid contents of individual plant extracts and plant mixtures. TF were found to be fairly high in plant extract mixtures than the individual ones. Among individual plants, *R. serpentina* revealed the presence of highest flavonoid contents (50.41±0.01mg CE/g) followed by *T. arjuna* (45.10±0.23mg CE/g), *C. oxyacantha* (40.32±0.0 mg CE/g) and *E. cardamomum* (33.90±0.10mg CE/g). Among individual plants and their combinations, 8th mixtures (T: R: C: Cr, 1:2:1:1) showed the presence of highest flavonoid (67.10±0.11mg CE/g) contents (fig. 2).

Table 1: Mixing ratios of different plants

Plant mixtures	Mixing ratios			
	T*:	R#:	C$:	Cr^
1	2:	2:	1:	1
2	2:	2:	2:	1
3	2:	2:	1:	2
4	1:	1:	1:	1
5	2:	4:	1:	1
6	2:	4:	2:	1
7	2:	4:	1:	2
8	1:	2:	1:	1
9	4:	2:	1:	1
10	4:	2:	2:	1
11	4:	2:	1:	2
12	2:	1:	1:	1
13	4:	4:	1:	1
14	4:	4:	2:	1
15	4:	4:	1:	2
16	Control			

[*]*T. arjuna*
[#]*R. serpentina*
[$]*E. cardamomum*
C. oxyacantha

Determination of antioxidant activity (AA)

Results of percentage inhibition of DPPH radical and inhibitory concentration (IC$_{50}$ values) are depicted in table 3. Among individual plants, *E. cardamomum* exhibited highest percentage inhibition (87.05±0.1%) followed by *C. oxyacantha* (81.05±0.03%), *R. serpentina* (80.15±0.07%) and *T. arjuna* (79.25±0.04%). Synthetic antioxidant (BHT) exhibited highest percentage inhibition as compared to individual plants. Natural antioxidant (ascorbic acid) showed percentage inhibition closer to individual plants. Among fifteen studied plant combinations, highest inhibition (92.0±0.09%) was shown by the 8th plant combination (T: R: C: Cr, 1:2:1:1) which was also greater than both standards and individual plants. Among individual plants, lowest IC$_{50}$ value was shown by *E. cardamomum* (0.164 mg/ml) followed by *C. oxyacantha* (0.216 mg/ml), *T. arjuna* (0.254 mg/ml) and *R. serpentina* (0.271 mg/ml). IC$_{50}$ values for BHT and ascorbic acid were 0.111mg/ml and 0.224mg/ml, respectively. Among plant combinations, lowest IC$_{50}$ value (0.109mg/ml) was also shown by 8th plant combination (T: R: C: Cr, 1:2:1:1) followed by 6th (0.135 mg/ml). Highest IC$_{50}$ value was shown by 13th combination (0.366 mg/ml). On the whole, highest percentage inhibition and lowest IC$_{50}$ value was shown by 8th combination.

Correlation between TPC, TF and AA

Correlation coefficients (*r*) among antioxidant activity, IC$_{50}$ value, phenolic and flavonoid contents are shown in fig. 3. A linear positive correlation was found between antioxidant activity, total phenolic and flavonoid contents. A negative correlation was observed among IC$_{50}$ value, total phenolics and flavonoid contents. The highest correlation coefficient was established between total phenolic and flavonoid contents among all correlation studied.

DISCUSSION

Green antioxidants are now getting attention due to their free radical scavenging potential. Medicinal plants are considered to be more effective when used in the form of multi-herb formulation. However, interactive actions between constituents in these multi-herbs and the underlying mechanism remain inadequately understood. Many reports are available on the phenolic, flavonoid contents and antioxidant activity of individual plant extracts but few researchers focused on synergetic antioxidant activity of combination of plant extracts.

The estimation of phenolics in plants has key importance for their use as medicine. Phenolics phytochemicals are secondary metabolites and largely disseminated in the plant kingdom. These compounds are very essential for plants due to their quenching ability because of the presence of hydroxyl groups (Elmastas *et al*., 2006). They exhibit different types of pharmacological properties: antioxidant, anti-inflammatory, antipyretic, antibacterial and anti-carcinogenic. In this study, plant combination exhibited considerably greater polyphenolic contents than individual plants (fig. 1). Reasonably high amount of total phenolics in plant mixtures may be due to coalescence of diverse phenolics from different plants (Maizura *et al*., 2011). Mixture of different plants with diverse phenolics may lead towards the formation of such an efficient mixture of phenolics which is responsible for maximum antiradical activities (Queiros *et al*., 2009). This study also revealed the presence of different types of phenolics (caffeic acid,vanillic acid, chlorogenic acid, p- coumaric acid, ferulic acid) with considerable concentration. These phenolic acids may be responsible for their free radical scavenging potential. Vanillic and chlorogenic acids have been found to have an inverse relationship with the incidence of various diseases. Earlier researchers determined phenolic acids in *T. arjuna* bark by HPLC analysis and found chlorogenic acid and ferulic acid in appreciable amount along with other phenolics (Jahan *et al*., 2012). From the medicinal plant extracts, separation of pharmacologically vigorous components remains a lengthy and tiresome process. However, Chromatographic methods are one of the easiest and most affordable methods for discovering plant components since these techniques are simple to run, reproducible and needs a miner equipment's.

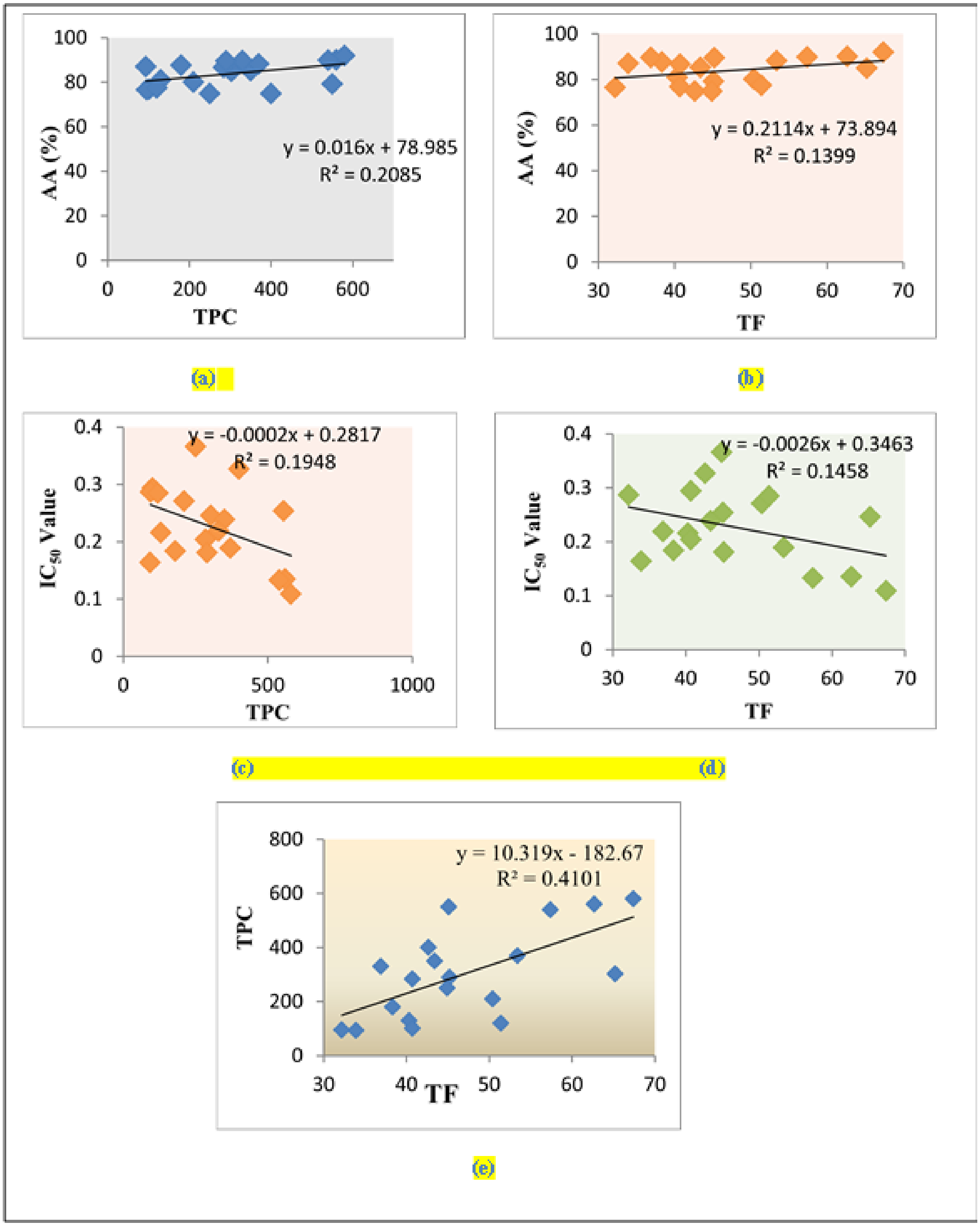

Fig. 3: (a) Correlation between Antioxidant Activity and Total Phenolic Contents. (b) Correlation between Antioxidant Activity and Total Flavonoid Contents. (c) Correlation between IC_{50} Value and Total Phenolic Contents. (d) Correlation between IC_{50} Value and Total Flavonoid Contents. (e) Correlation between Total Phenolic and Flavonoid Contents.

Table 2: Concentration of different phenolic acids in plants determined by HPLC fingerprinting

Phenolics compounds	Concentration in Plants (ppm)			
	T. arjuna	*R. serpentina*	*C. oxyacantha*	*E. cardamomum*
Caffeic Acid	-	25.58	23.26	61.3
Vanillic acid	-	-	-	73.8
Chlorogenic acid	271.63	-	24.09	-
p- coumaric acid	-	27.46	15.68	-
Ferulic acid	101.69	-	93.28	143.73

Table 3: Percentage inhibition of DPPH radical and IC_{50} value by different medicinal plants and standards

Plants & standards	%age inhibition of DPPH radical at different concentrations (mg/ml)					IC_{50} Value (mg/ml)
	0.2	0.4	0.6	0.8	1.0	
T	75.15±0.3	75.85±0.1	76.95±0.01	78.7±0.02	79.25±0.04	0.254±0.01
R	72.0±0.04	75.9±0.2	76.55±0.08	76.65±0.2	80.15±0.07	0.27±0.04
Cr	78.95±0.1	79.4±0.05	80.0±0.4	80.15±0.1	81.05±0.03	0.216±0.03
C	83.35±0.3	85.2±0.06	86.1±0.01	86.9±0.07	87.05±0.1	0.164±0.02
AA	78.05±0.4	78.85±0.1	78.95±0.02	80.2±0.02	80.8±0.08	0.224±0.03
BHT	90.1±0.01	91.15±0.2	91.2±0.03	91.45±0.1	91.8±0.04	0.111±0.02
1	75.45±0.12	75.9±0.05	78.5±0.13	82.6±0.28	84.95±0.37	0.246±0.07
2	79.75±0.20	81.0±0.13	81.2±0.07	85.7±0.03	86.75±0.56	0.204±0.05
3	81.7±0.32	81.9±0.03	85.8±0.01	86.3±0.23	87.7±0.08	0.184±0.021
4	74.35±0.19	77.6±0.08	81.85±0.14	82.15±0.2	85.3±0.01	0.239±0.031
5	76.55±0.1	80.15±0.07	83.05±0.16	85.8±0.09	89.7±0.07	0.219±0.03
6	87.1±0.02	88.05±0.01	88.65±0.03	88.9±0.4	90.1±0.04	0.135±0.06
7	87.55±0.12	87.9±0.05	88.6±0.01	89.35±0.5	89.95±0.03	0.133±0.033
8	90.25±0.24	90.6±0.09	90.8±0.02	91.1±0.98	92.0±0.09	0.109±0.011
9	81.85±0.06	83.35±0.04	84.3±0.09	87.3±0.2	89.55±0.01	0.181±0.012
10	80.8±0.4	82.35±0.1	84.9±0.07	86.75±0.1	88.3±0.7	0.189±0.061
11	72.1±0.32	72.85±0.01	72.9±0.05	73.7±0.07	76.9±0.4	0.294±0.02
12	69.25±0.06	69.55±0.01	70.0±0.81	73.9±0.06	74.9±0.3	0.327±0.03
13	63.5±0.01	67.15±0.02	68.95±0.90	71.15±0.2	74.95±0.01	0.366±0.07
14	70.7±0.45	73.45±0.9	76.15±0.21	76.25±0.3	76.6±0.4	0.287±0.02
15	70.5±0.02	74.4±0.31	75.75±0.4	76.95±0.6	77.45±0.21	0.285±0.05

Data are mean (n = 3) ± SD.

Flavonoids belong to a group of poly-phenolic compounds that are regarded as powerful antioxidants. Molecular structure of flavonoids especially the presence of hydroxyl group is responsible for their antiradical capabilities (Kiranmai *et al*., 2011). Flavonoids with a particular structure and hydroxyl location in the molecule can only serve as proton donors and exhibit radical attenuation potential. In general, plant mixtures exhibited comparatively large quantity of TF. The plant 8th combination (T: R: C: Cr, 1:2:1:1) exhibited highest amount of phenolics and flavonoid contents. Thus, it was expected to express the highest antiradical potential due to correlation of phenolic and flavonoid with antioxidant activity as reported by earlier researchers (Olajire and Azeez, 2011; Borkataky *et al*., 2013). The herbal combinations at specific ratio showed positive synergism and their antioxidant potential was higher than the individual plants.

Evaluation of antioxidant potential of natural products has been of key importance due to injurious effects of free radicals in biological systems. Free radical attenuation ability is well known method of antioxidant to prevent oxidation. The DPPH (2, 2-diphenyl picryl hydrazyl) radical examination is extremely well-known mode to assess antioxidant species since the reaction potency can be observed spectrophotometrically (Khatun *et al*., 2011). IC_{50} values symbolize the amount/concentration of antioxidant that causes decolourization/neutralization of 50% DPPH radicals (Ayesha *et al.,* 2013). Lowest IC_{50} value indicates dominant free radical inhibitory effect. It is calculated from linear regression equation from the plot of concentration against percentage inhibition (Rasool *et al*., 2011). Sixteen herbal combinations from four medicinal plants were made and their synergetic antioxidant potential was assessed. Among the different combinations, 8th mixture exhibited the highest free radical scavenging activity with lowest IC_{50} (table 3). This combination also showed enhanced antioxidant potential than individual plants. Antioxidant potential of mixture (8^{th} T: R: C: Cr, 1:2:1:1) was much better than synthetic and natural standard antioxidants. Highest phenolics and flavonoids were also observed in 8^{th} plant mixture. Its lowest IC_{50} and higher antioxidant potential might be due to positive interactions and good combination of phenolic and flavonoid contents. Synergism might occur through interaction between each constituent of individual plants with diverse pharmacological functions. In this way bioactive components which are less potent independently may turn therapeutically more active when used in combination (Xh *et al*., 2009; Xu *et al*., 2014). A linear positive correlation was observed between antioxidant activity and total phenolic, flavonoid contents indicating them the main antioxidant compounds. The results of this study evidently reflected that plant combinations possessed greater amount of phenolics, flavonoids and antioxidant activity as compared to individual plants which is certainly due to synergistic effect.

CONCLUSION

It was concluded from current study that plant extracts when used in combination showed better antioxidant potential than individual plant extracts. Phenolic and flavonoids present in a mixture can interact, and their interactions can influence the total antioxidant ability. The highest synergistic antioxidant activity was shown by extract mixtures of *T. arjuna*, *R. serpentina*, *E. cardamomum* and *C. oxyacantha* plants with the ratio of 1:2:1:1. Thereby, mixing of plants understudy with given ratio can bring better effects against free radical progression diseases. These results can provide some basis for the supposed synergistic effects of traditionally used plants, and make possible their use in combination as functional foods and dietary supplements.

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